Proteomics : Principles And Techniques
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Module No.# 14
Lecture No. # 14
Gel-Based Proteomics
Two-dimensional electrophoresis Workflow:
Staining and ImageAnalysis

So, welcome to the Proteomics course.

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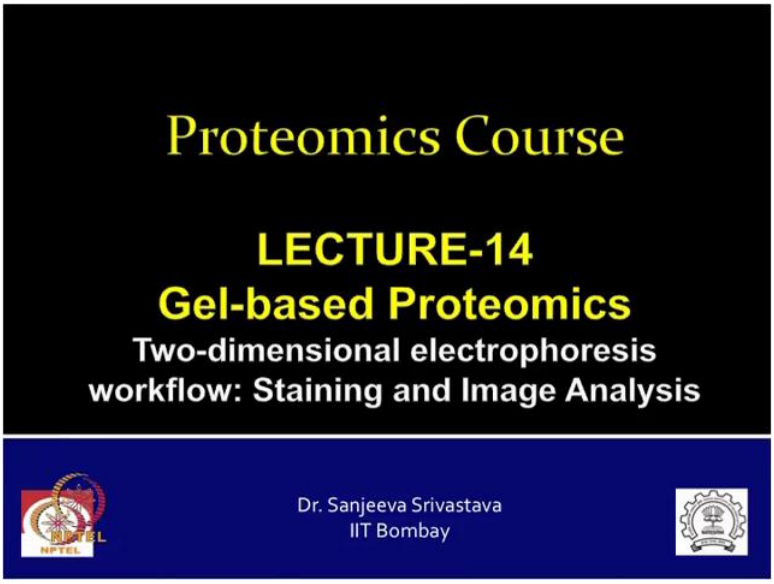

In the last lecture, we started discussing about the gel-based proteomics. How different techniques are used to separate proteins, the complex protein mixtures and I mentioned that SDS page, blue native page, two dimensional electrophoresis and various advanced forms of gel-based proteomics; they all together help to study different type of problems by applying gel-based approaches in proteomics.

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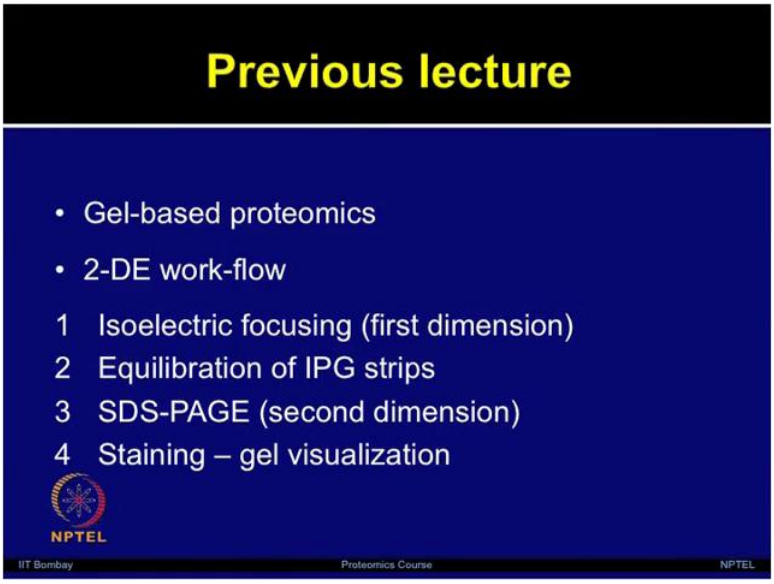

We started discussing about the work flow of how to perform two dimensional gel electrophoresis, I gave you a step wise method in which the first step was to do the isoelectric focusing. Second step equilibrating the immobilized pH gradient strip to make it compatible for the second dimension separation in SDS page, third step was SDS page to separate proteins in molecular weight, fourthwould like to visualize your protein spots on the gel, so staining methods. In the last lecture, we discussed about two staining methods, one was Coomassie brilliant blue, other silver staining.

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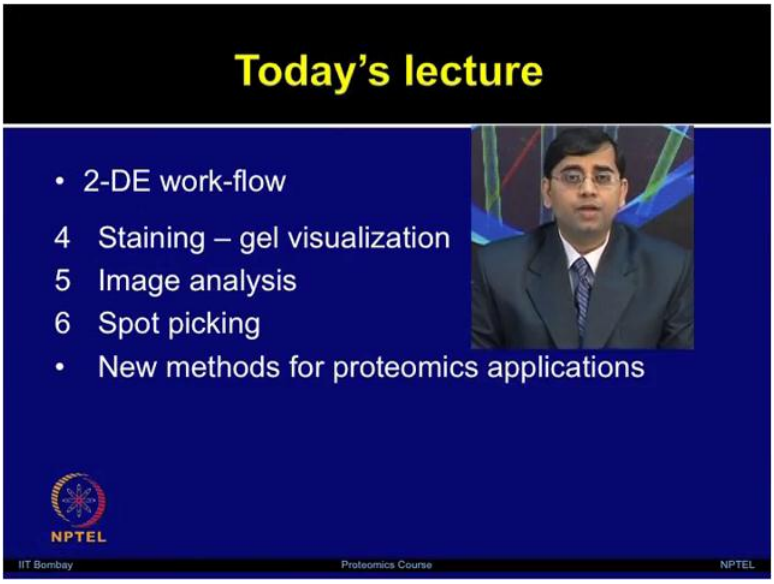

So, now in the same work flow,today I will continue on different type of staining method. I will describe you some more sensitive stains and stains used for different applications such as sypro ruby, Pro Q diamond, cyanine dyes, each one of these are used for various unique applications.For example, the cyanine dyes, they are used in one of the advanced form of gel electrophoresis which is known as difference in gel electrophoresis. In this method, you mix the Cy dyes together and separate the proteins on one gel itself. Sypro ruby is one of the very sensitive stain and this stain is also used for looking at the different type of protein patterns such as it can be used for (( )) staining.

Now, if you want to look at post transition modification, few stains such as Pro Q diamond can be used, but if you want to look at the overall protein pattern, then you can do dual staining with sypro ruby or some other stain.

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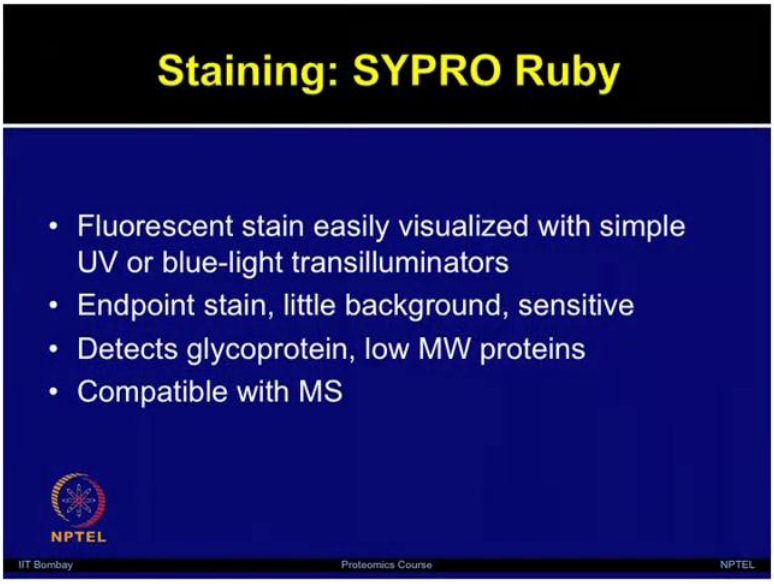

So, let us continue our today's lecture on different type of staining methods being used. So, let us first start with sypro ruby staining, this is one of the fluorescent stain, which is usually visualized on the gel in the UV or blue light transilluminators, this stain very sensitive and it is endpoint stain. So, very little background is used, unlike the silver stain where there is lot of issues with the very dark background,the sypro ruby has overcome those limitations.

This is also one of the very sensitive stains and it can also be used for studying different type of post translational modification such as glycoproteins, often even low molecular weight proteins which are low abundance protein, those can also be visualized by using this type of a stain. So, sypro ruby is one of the very useful stains, which is very compatible for doing further processing with the mass spectrometry. So, if you want to separate the proteins, you have to visualize them. After that your end aim is to characterize those proteins, what those proteins are, and further you want to do experiments on those. In that light, sypro ruby is very compatible for mass spectrometry and you can further characterize yourproteins.

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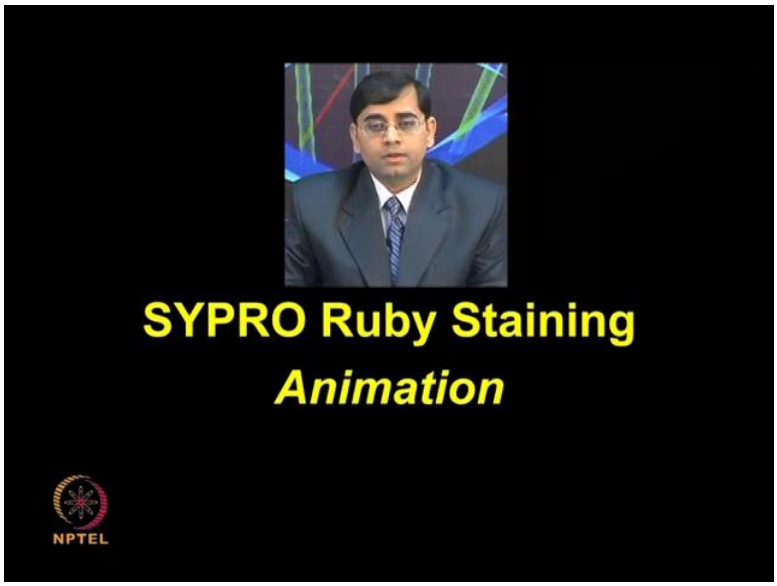

So now, I will show you the steps involved in performing sypro ruby staining, so I will show you one animation.

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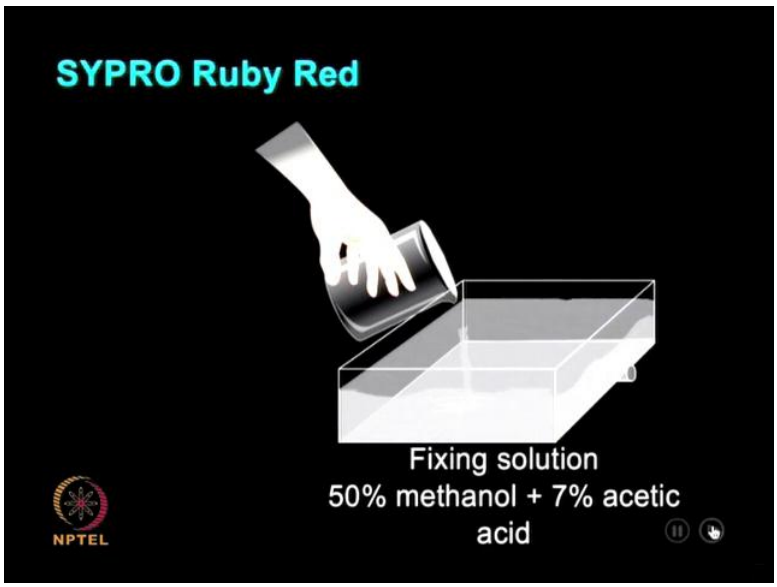

Sypro ruby red, the completed gel is first placed in a fixing solution of methanol and acetic acid that fixes the protein bands in the gel and minimizes any diffusion.

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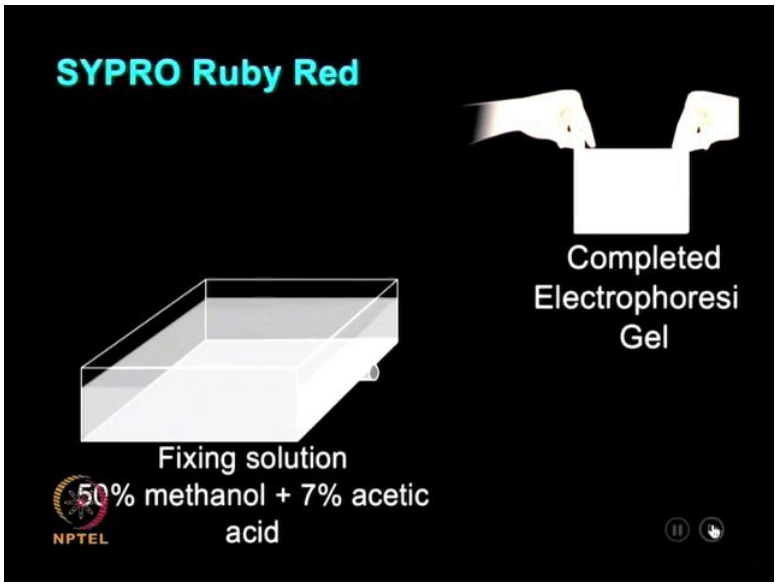

This must be subjected to gentle shaking for around half an hour.

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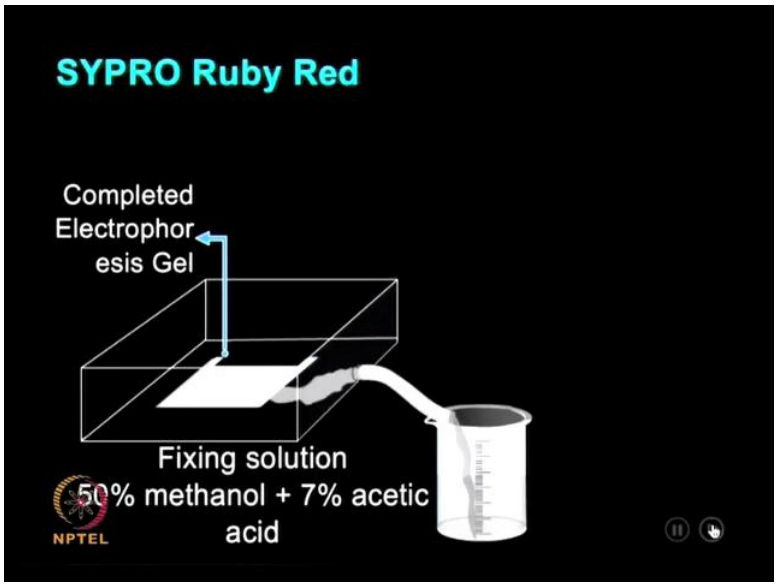

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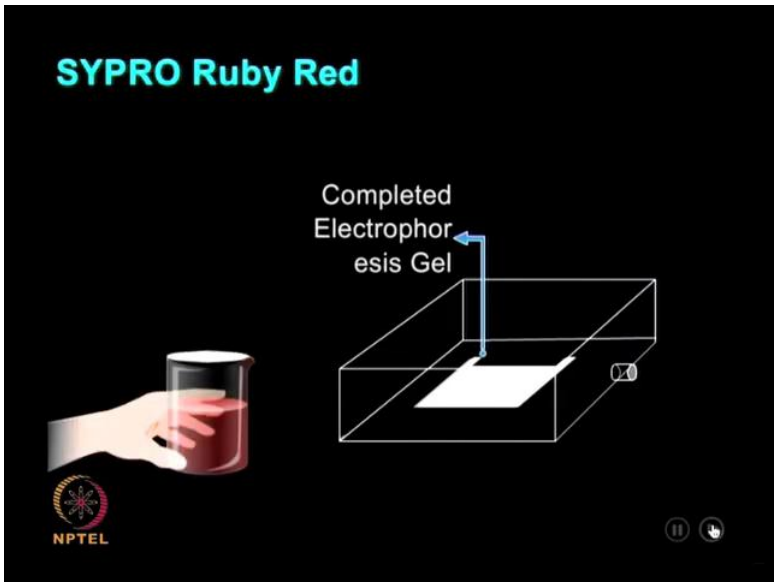

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After fixation step, the gel is soaked in the fluorescent sypro ruby red stain solution. Gel should be kept on the mechanical locker for uniform shaking. During the process, the gel gets uniform staining with the (( )) based (( )).

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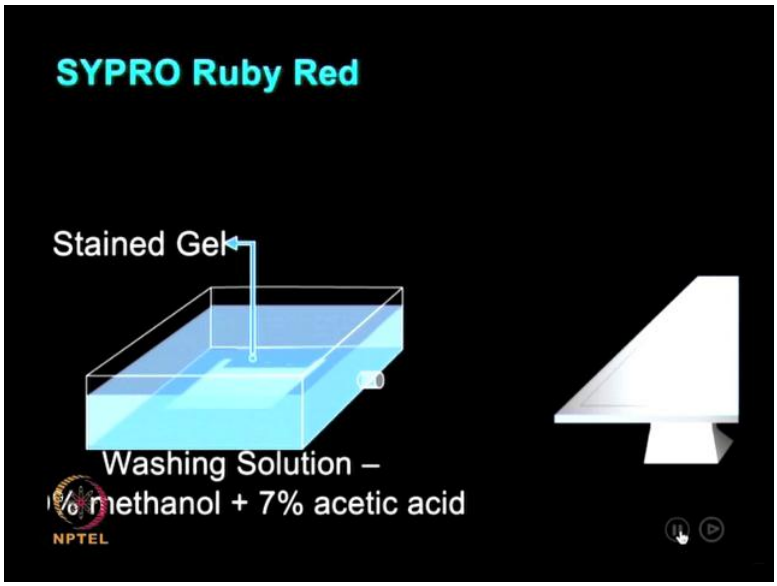

After this overnight step of staining, the gel can be washed with methanol and acetic acid solution, again you need to keep it on rocker so that uniform destaining can be performed.

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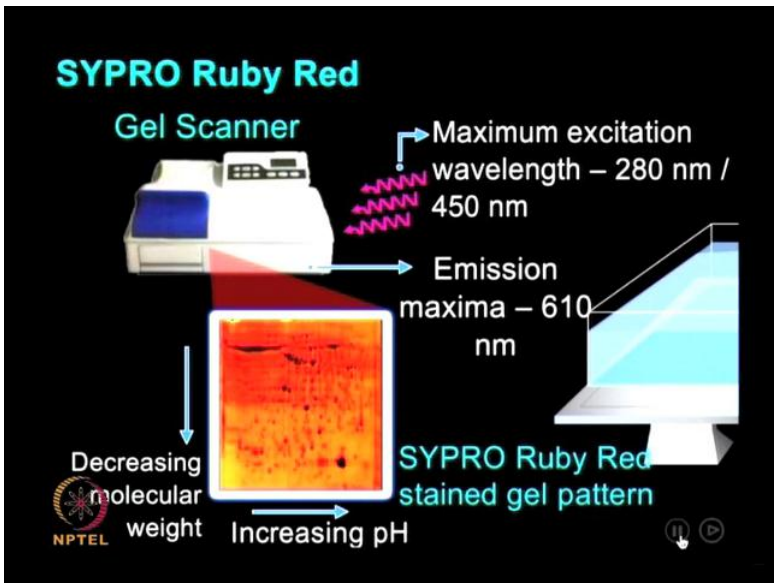

The MS compatible gel is then viewed using UV or laser scanning instrument with an excitation wavelength near 280 or 450 nanometres, this stain has two excitation maxima and emission maxima of 610 nanometre. In this animation, you can see a gel pattern which is showing you the staining obtained after sypro ruby red and it is showing separation in two dimensions based on the molecular weight and isoelectric point.

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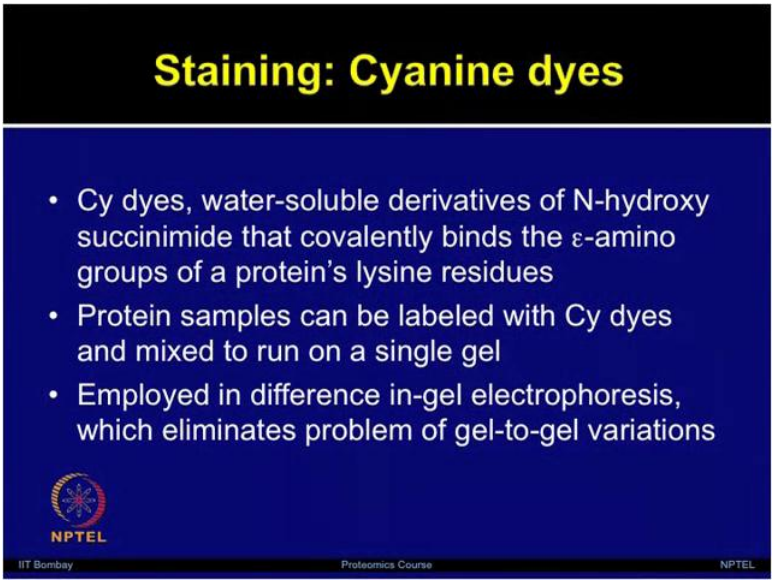

So, now after looking at this animation,let us continue on second staining method which is use of cyanine dyes. As I mentioned, theses are very sensitive dyes and these are water soluble derivative of n-hydroxy succinimide,which is covalently bound to the alpha amino groups of a protein's lysine residue. Now, since this labelling is minimal labelling, your overall massis not going to change by adding different type of Cy dyes such as Cy 2,Cy3 and Cy 5, protein samples can be labelled with these dyes and then mix together to separate the proteins on one gel.

Now, unlike your two dimensional electrophoreses where you have two gels separately, you are comparing a control and a treatment gel and those two are done separately; in this case, whole sample is run on one single gel. Now, when you are separating everything on one gel and just you are changing the fluorescence scanning parameter, then there is no variation overall and at the end you can obtain from the same gel three different images based on the emission, fluorescence and etcetera. So, this method is implied in difference in-gel electrophoresis which is one of the very advanced form of two dimensional electrophoresis and it eliminates many gel to gel variation problems, I will describe this technique in much more detail in the later part of the course.

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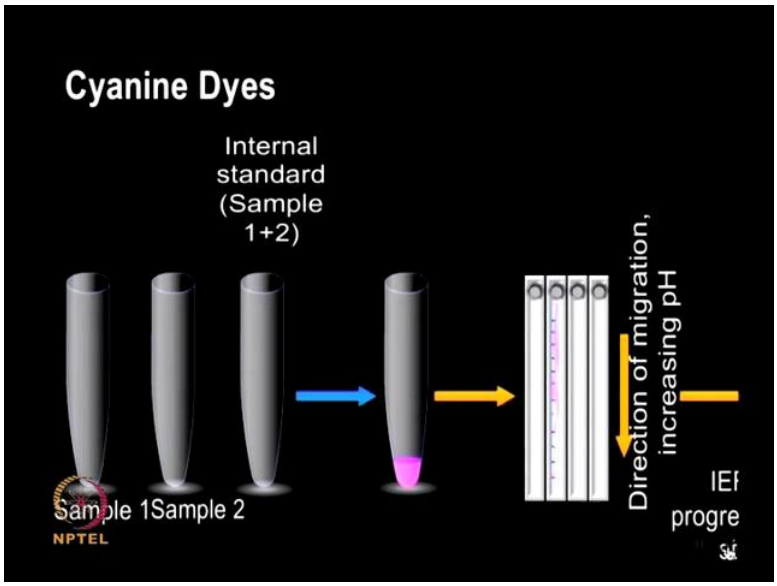

But briefly I will show you this animation for cyanine dye staining. Cyanine dyes, in this detection technique, the dyes are mixed with the samples prior to the electrophoresis , the first sample was labeled with Cy3 dye and the second sample is now mixed with Cy 5 dye.

Now, an internal pool made from both sample 1and 2 is labeled with third dye which is Cy 2 dye,each protein sample as well as the standard internal pool is labeled with a differently florescent cyanine dye which allows all the protein samples to be simultaneously run on a single 2 D gel. After dye labeling and mixing all the samples together, the isoelectric focusing can be performed followed by the second dimension separation in SDS page.

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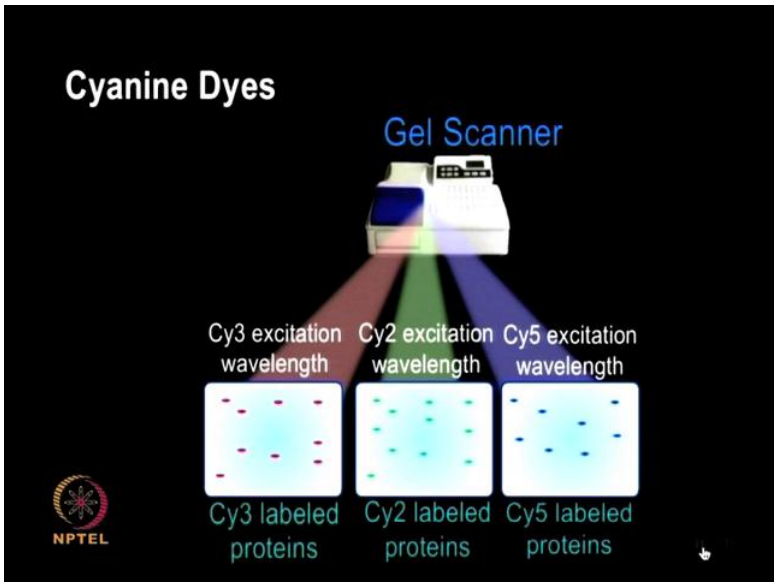

The gel can be further scanned and viewed by eliminating it alternately with excitation wavelength corresponding to the various Cy dyes, this technique minimizes the gel to gel variations and allows a large number of samples to be run by using lesser number of gels, therefore it provides very useful solution to various proteomic applications.

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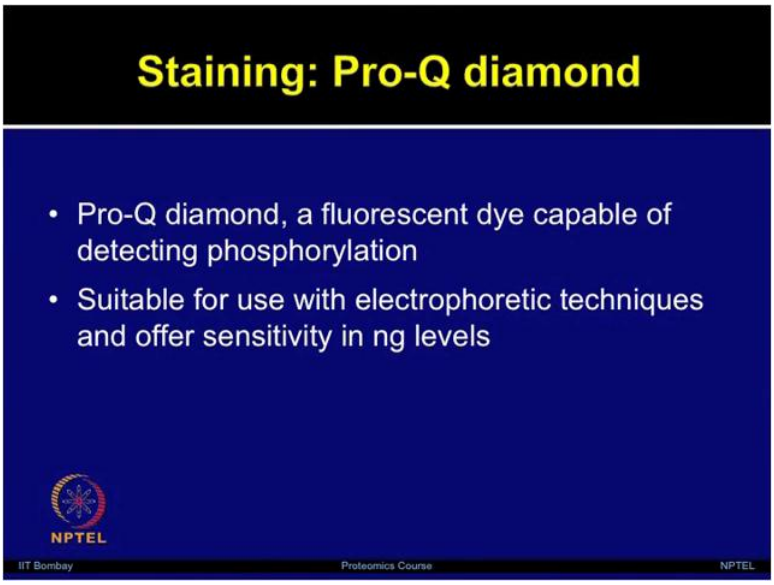

So, now after looking at this animation, you are familiar that there are different type of staining methods can be used. Now, let me introduce you to another stain which is Pro Q diamond which is used for studying phosphorylation of the post translationally modified

proteins, now Pro Q diamondthat is dye, fluorescent dye which is capable of detecting phosphorylation.

So, if your proteins having some phosphorylation, addition of some phosphate residues, now this stain is quiet sensitive to pick up those patterns of phosphorylation or post translational modification in your gel, this is very suitable for use in electrophoresis and it offers sensitivity in Nanogram level. Now, let us say you want to look at the post transitional modified forms of the protein, but after that you would also like to know which proteins it belong to, we would like to do the mass spectrometry on these.

So, often it becomes very difficult if you just done the Pro Q diamond on one gel, so you would like to do the dual staining. So, first you would like to detect only your post translationally modified form of the protein or the first violated forms, then followed by the same gel can be used for further staining with some end point stain such as sypro ruby. Now, you take the same gel, now further stain with the sypro ruby, so you will obtain two images for the same gel where few spots can show the PTM form and the overall, the global pattern of the proteins can be visualized on different gel. So, in this regard by comparing the PTM form or the phosphorylated form along with the complete profile of the gel, one can obtain the very comprehensive picture, this many proteins are separated on the gel, among those certain numbers are phosphorylated, one can then further excise those spot and do the mass spectrometry for further identification andcharacterization.

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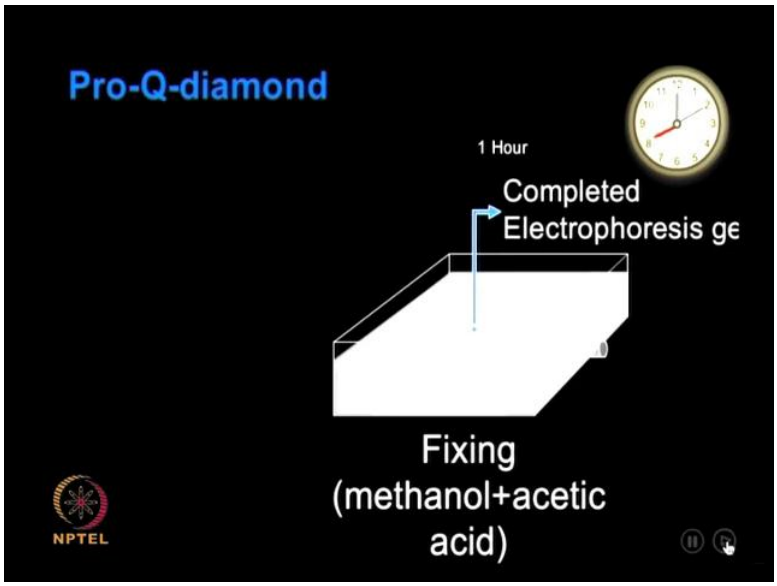

Now, let me show you this animation for doing Pro Q diamond staining followed by dual staining. Pro Q diamond, this is very useful staining technique for detection of phosphoproteins and when used in combination with sypro ruby red, it also provides a very comparative profile of total protein contents and the phosphoproteins content.

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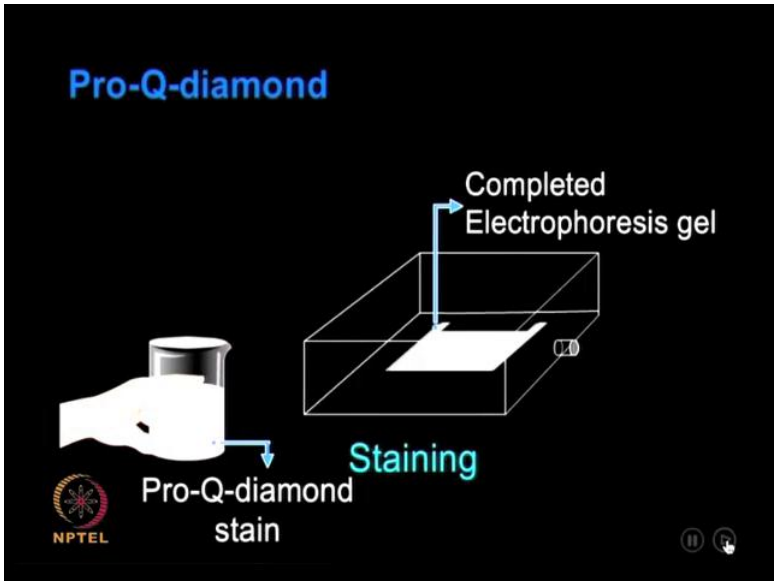

The first step is to perform the fixation and followed by addition of Pro Q diamond stain, the staining procedure is similar to the sypro ruby red which was earlier described.

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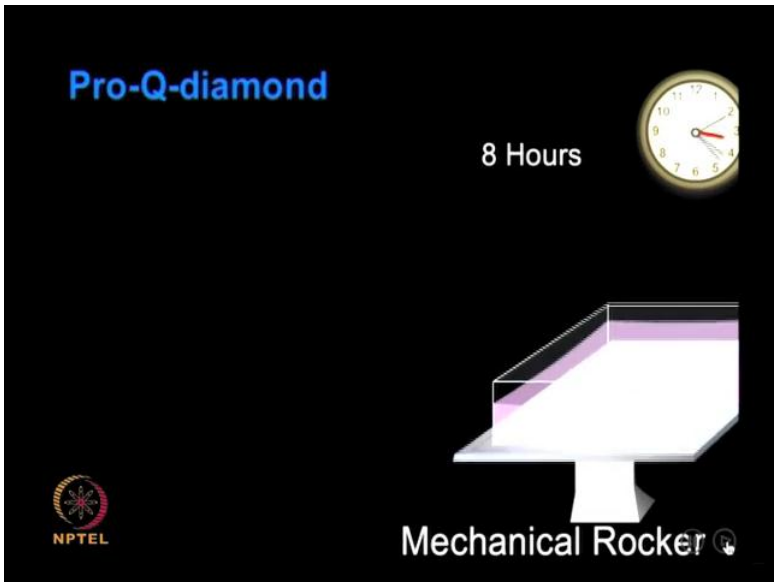

This fluorescent dye offers more sensitivity down to Nanogram level and detects specifically, the phosphorylation taking place at serene the anine or tyrosine residues; for uniform staining, you need to add the staining solution for at least eight hours or overnight.

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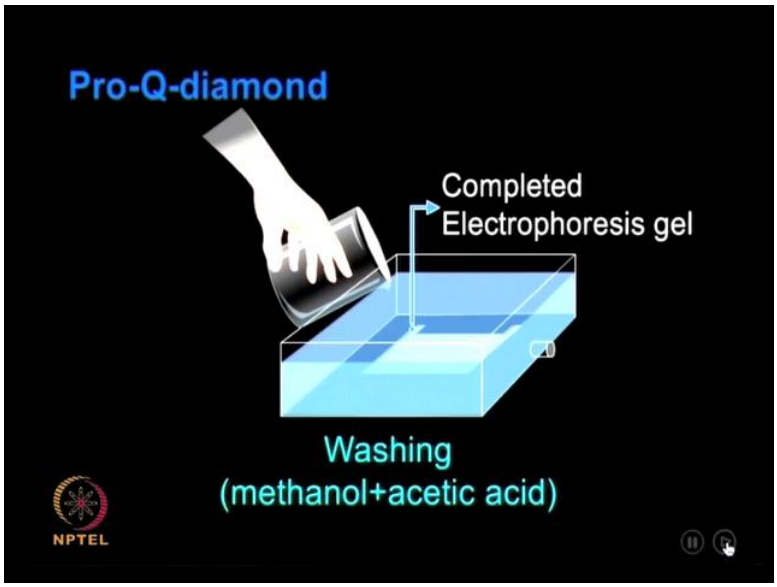

Now, staining solution can be removed and further wash with methanol and acetic acid.

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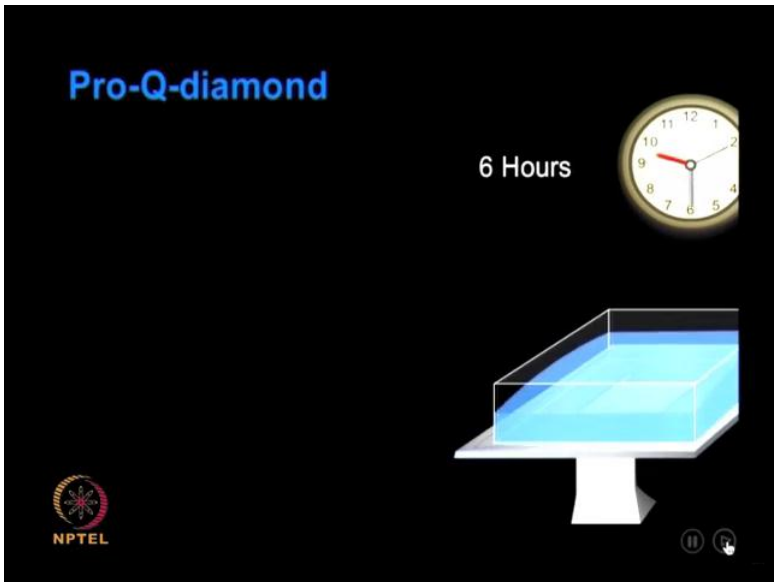

For uniform destaining, it is good idea to keep it on gentle shaking for at least 6 hours.

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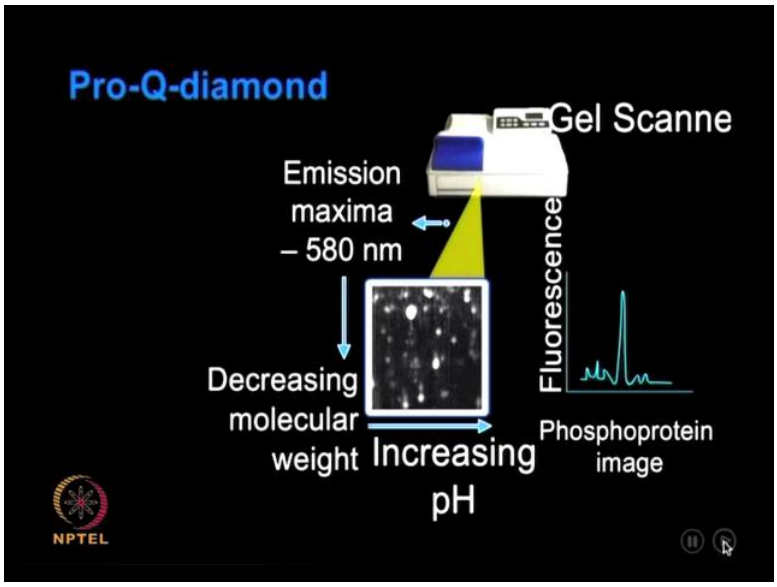

Gel can be further scanned and image can be procured, especially at emission maxima of 580 nanometre and it provides again on the 2 D, two dimensions decreasing molecular weight and increasing pH in formation for all of this protein spots.

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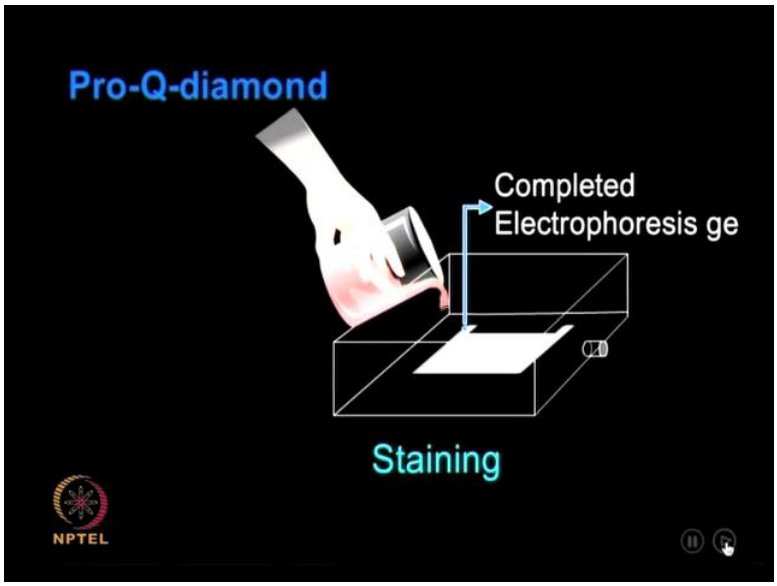

Often people combine this phosphor stain along withsypro ruby stain to obtain dual staining information which was described in the lecture. So, in the animation you have seen that how Pro Q diamond can be used to detect post translation modified form or the phosphorylated form of the proteins.

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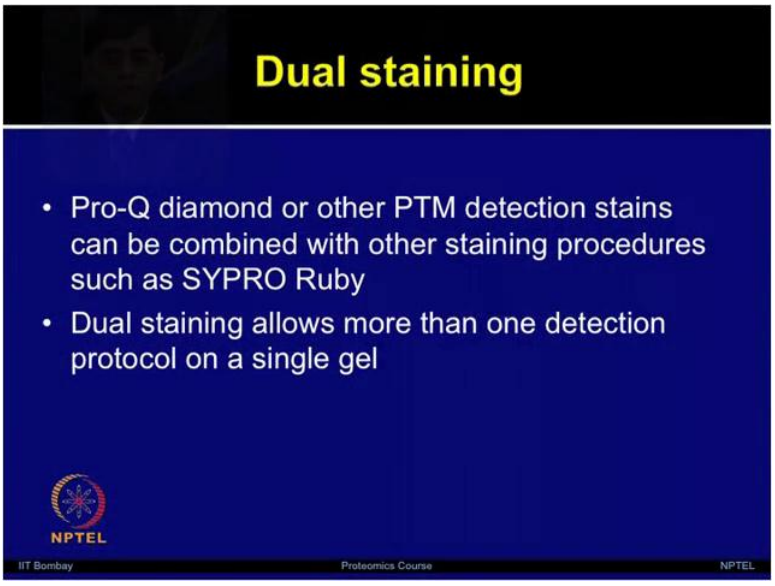

And then other staining procedure such as sypro ruby can be used for dual staining, now dual staining allows more than one detection protocol on the same gel. So, it has lot of merit and I hope after watching this animation, you must be able to appreciate that.

Suppose, discussing about different type of staining procedure which are available. Now, you have separated the protein spots on the gel, now we have visualized that by using different type of stains, again theselection of stain depends upon the type of application you are looking for; often you have limitations of your protein, if you did not have enough protein, you probably used silver or sypro ruby, if you had enough protein you went with Coomassie brilliant blue staining, you are interested in looking certain form of PTM, you used Pro Q diamond or other specific stains.

Now, if you want to do some more advanced form to eliminate lot of gel artifacts, probably we used cyanine dyes. So, after all of this type of stainingdetection, now you are ready to scan the image, because now you can see all the protein spots available on the gel in different colors, whether it is blue in Coomassie or brown, in silver pinkish, in sypro or different type of florescent pattern in cyanine dyes and Pro Q diamond.

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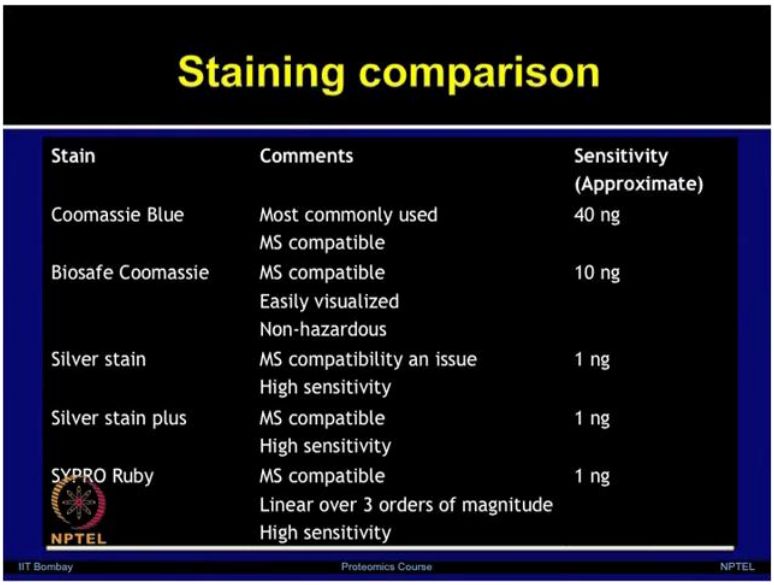

So, now let me describe you some staining comparison available for various type of stains which we discussed, before we move onto the next part on the image scanning. So, in a net shell, here you can obtain information for various stains which are available, their properties and their sensitivity; please note, this sensitivity is only approximate. So, Coomassie blue that is one of the most commonly used stain which is employed in most of the laboratories word wide.

It is compatible for mass spectrometry, sensitivity is in the range of 40 to 100 Nanograms, bio safe Coomassie that is also MS compatible, another advanced form of Coomassie staining which isenvironmental friendly, it is non hazardous and it offers sensitivity in the range of 10 Nanograms. We have talked about silver stain, when you have low protein in your extract, probably silver stain is a method of choice or sypro ruby; in that case silver stain is very sensitive, it offers sensitivity in the range of one Nanogram, it is having some issues with mass spectrometry compatibility.

But people have overcome those limitations by changing certain reagents and now there are advanced form of silver stain available such as silver stain plus which are sensitive as well as compatible for the mass spectrometry. Next, we will talk about sypro ruby, this is again a very sensitive staincompatible for mass spectrometry and it gets very low background on the gel. So, it offers linear order of three orders of magnitude. So, you can see your signal in much clear, white background. So, by looking at this table overall, you can compare different type of stain available and which one you would like to use for your application.

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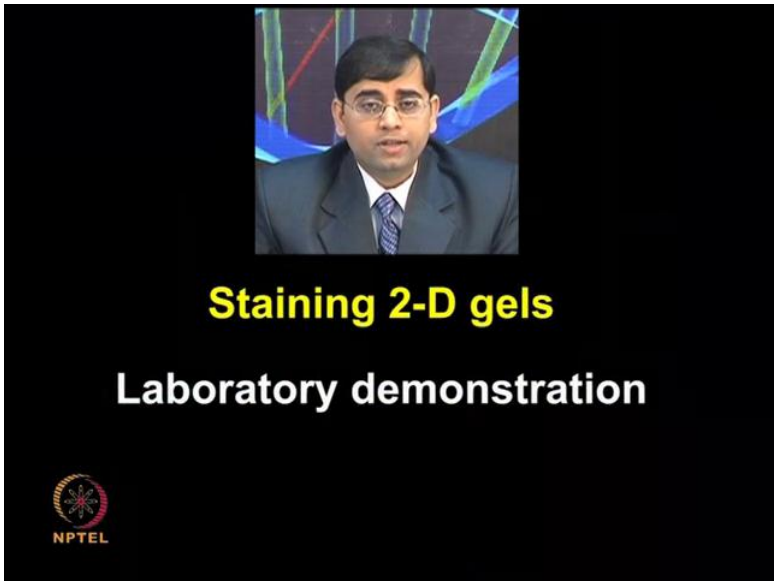

Next, I will talk about laboratory demonstration of staining two dimensional gels. So, in this video, I will show you, how you can use Coomassie or silver stain to stain your 2 D gel and then, what else steps you need to perform to obtain this pattern and finally, what type of imageyou can acquire after the staining.

Gel staining and de staining, this process involves removal of the gel from the electrophoresis assembly followed treatment with a fixing solution, staining solution and finally the destaining solution. Gel removal and protein fixation; soak the gel present between the glass plates in fresh distilled water to remove any bound SDS. Pour the fixing solution containing methanol and acetic acid into another tray, open the glass plates and carefully remove the IPG strip from the gel, then transfer the gel into fixing solution by gently sliding it off from the glass plate, place the tray on a mechanical shaker for an hour to ensure that the protein sports gets fixed onto the gel thereby minimizing any diffusion.

Gel staining, transfer the gel carefully into another tray containing the Coomassie blue staining solution and place it on the shaker to ensure uniform contact of the gel with the solution, the negatively charged Coomassie dye interacts with protein through ionic and other non covalent interactions, there by staining them with a blue color.Add some more staining solution such that the gel is completely immersed and leave it over night for around 8 to 10 hours.

Destaining, drain out the staining solution from the tray and pour the destaining solution consisting of methanol and acetic acid into the tray, place the tray on the shaker for around 6 to 8 hours until the background stain of the gel is completely removed and the spots are clearly visible. Another commonly employed technique for visualization of protein bands is the use of a silver staining solution; in this procedure, the gels are first treated with the fixing solution followed by the silver stain and finally with a developing solution, where the silver ions get reduced to metallic silver by an alkaline solution of formal dehyde and sodium carbonate, the dark protein bands can be viewed well against a light background.

Here, we showed gels containing separated proteins from serum bacteria and plant samples that have been stained with Coomassie blue dye as well as plant protein sample that has been stained with silver dye. So, after looking atthe laboratory demonstration, now you are clear that, what are the steps involved in doing the staining procedure. Now, in this image which is a two dimensional image obtained from human serum, you can see the molecular weight and isoelectric point of proteins separated in two dimension, the first dimension is isoelectric point, second dimension is the molecular weight. So, for

each of the protein spot, you can obtain both information for isoelectric point and molecular weight, this is a gel with the Coomassie stain.

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So, now let us see how we acquire these images and what type of the scanning methods available, soimage analysis is another one of the very important aspect of two dimensional electrophoresis workflow.

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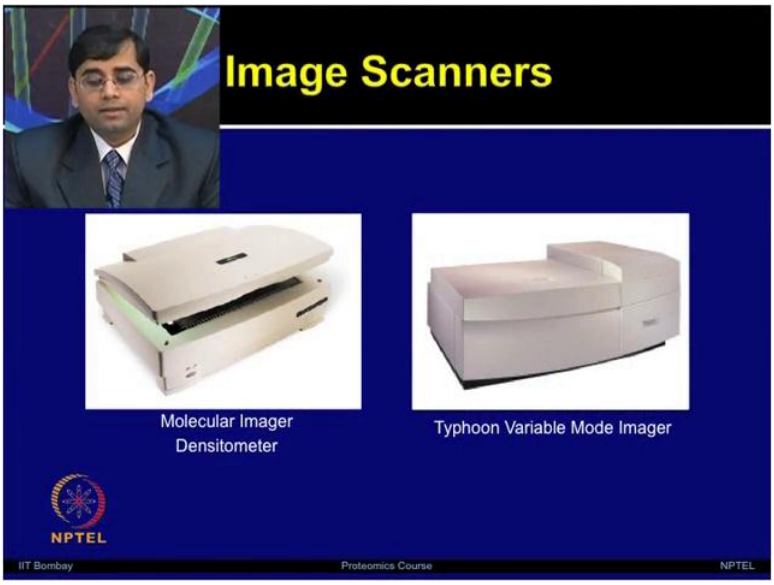

There are different types of image scanners available from different vendors such as, one I have shown here molecular imager densitometer, other the typhoon variable mode imager, there are many staining image scanners available.

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So, now, how to analyze these images, do you want to the things manually, so can you take your gel patterns and sit twoof you together and say,this is my protein in control, this is your protein in treatment, now I am going to look at each spot manually and then going to size the spot based on this comparison. So, that is going to be very very tedious work and you will not have any information about the, whether your spot shaving any statistical significance or not, how reproducible those are. So, you need to scan it by using good scanners and then finally, you need to analyze your image from different softwares which are available.

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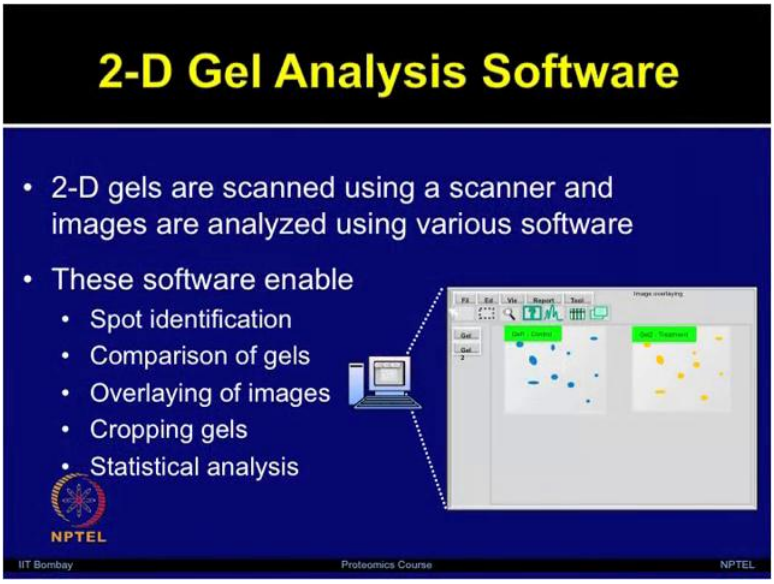

So, there are commercially many softwares which are available for doing twodimensional gel analysis, I will give you comparative table at the end, but almost all of these software, they take this scanned images and analyze your gels by using various steps. So, all these software enable the spot identification, comparison of the gels, overlaying of the images from your control and your treatment, copying the gel, the part of which you want to compare and further doing the statistical analysis.

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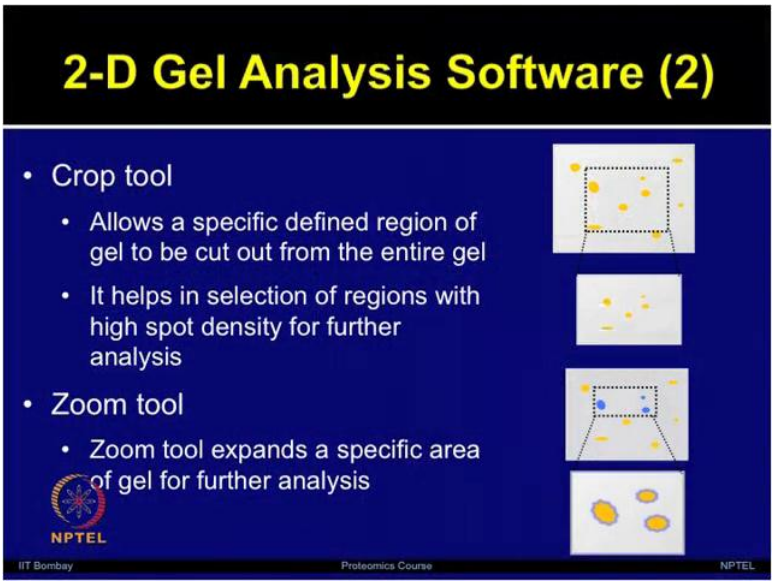

So, the crop tool that is the first part which you would like to use, if in your gel you have some extra regions which, where you do not have any of the sport of interest, probably you would like to crop those regions and crop both your control and treatment just uniformly.

So, this crop tool allows for a very specific defined region of the gel to be cut from the entire gel, it helps to select the region with the high spot density which can be used for doing further gel analysis, next you would like to see your spots in more detail. So, you would like to use zoom tool which can expand a specific area of the gel for doing further analysis.

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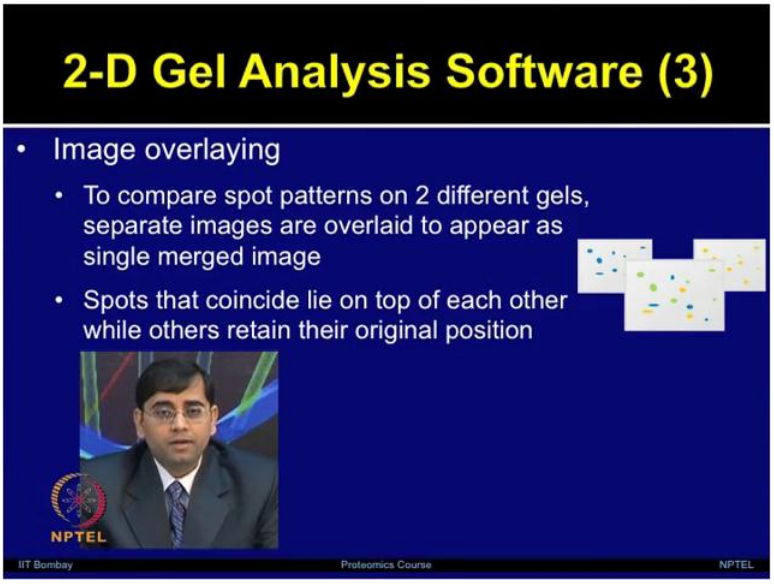

Now, image over laying is very important aspect, because if you are comparing two gels, on one you have control, other you have treatment, you would like to overlay those images together to compare their spot pattern present on two different gels. Because you have acquired two separate images, now you need to overlay those so that it can appear as a single merged image.

Now, spots which are going to coincide on top of each other where as you can also locate their original position from the each of the individual images. So, image overlaying is important aspect where you can merge your control and your treatment gels.

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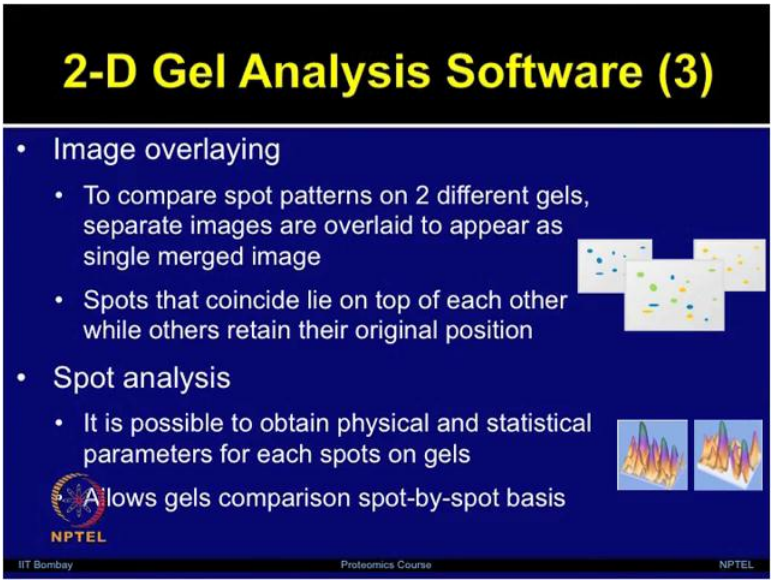

Then you would like to do the spot analysis where it is possible that you can obtain physical and statistical parameters for each spot which is present on the gel, you can look at the 3 dimensional views of each of the spot, how they are differing from the control to the treatments and then, you allow the comparison of the gel spot by spot basis. So, often running a gel or acquiring images and generating lot of data is much straight forward as compared to doing the analysis which is more tedious step, one has to release it and go through the gels, usually spot by spot to analyze the gels.

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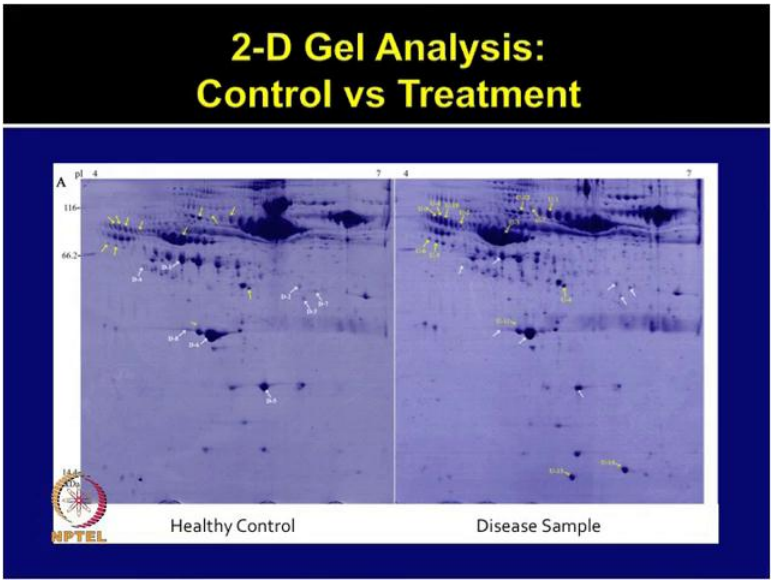

Now, I am showing you gel pattern of control and its comparison with the treatment gel, these are taken from one of the healthy control and disease sample and each of the spot is compared from the control to the treatments. And one can look at from different healthy controls and different disease sample, what is happening to each of this spot and if there a statistical significance for their overall change, if it is going up or going down is that uniform in all the gels and how significant that is. So, all of this analysis can be performed by using different softwares.

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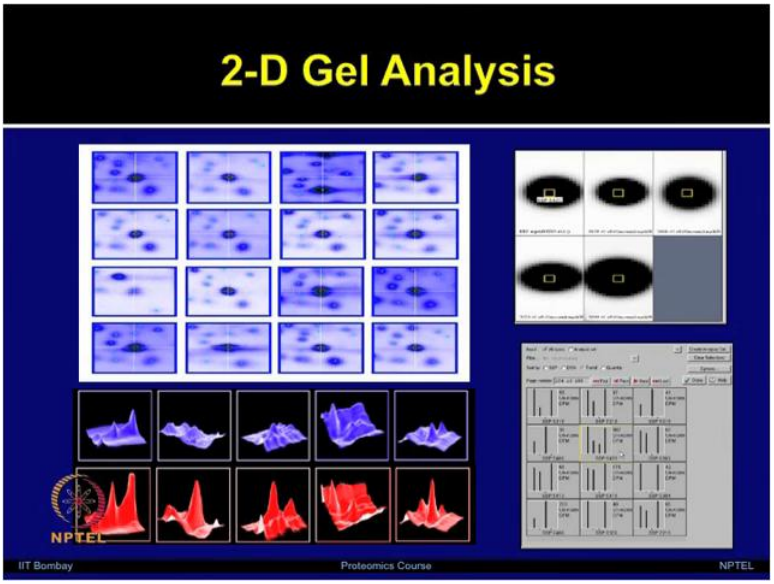

As you can see in this image, I am showing you one spot which you would like to compare across 16 gels, now you have zoomed into that particular region and further you are looking at the three dimensional pattern of each of the spot, how they are different from the control shown in the black and the blue spots and the treatment which is shown in the, on the black background in the red spot.

So, after looking at three dimensional views of this particular protein, you can confidently say that this protein expression is changing and it is going a higher amount in the treatment. Now, one can look each ofthis spot intensity in much detail and then followed by plot, different type of parameters for percentage volume or spot intensity to compare their values and do the statistical comparison.

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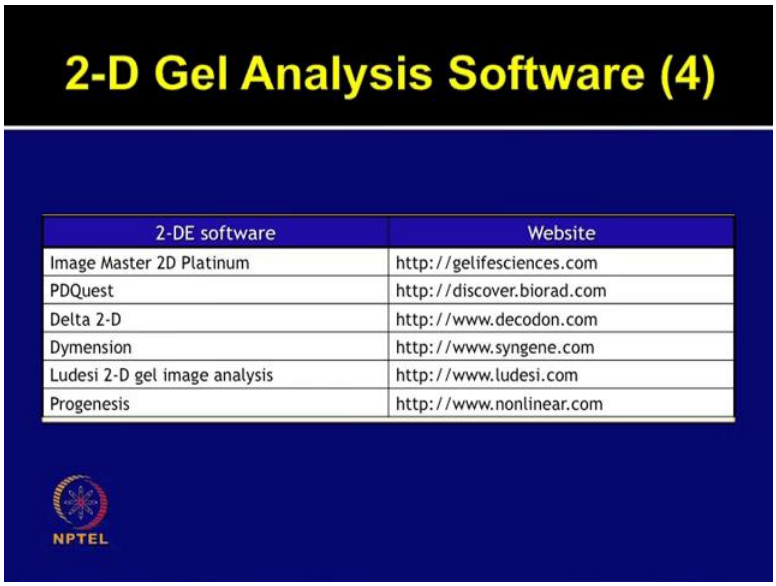

There are various commercial softwares which are available for comparing the 2 D gels such as image master 2 D platinum, from ge life sciences, PD Quest from biorad, Delta 2 D from decodon, Dymension from syngene, Ludesi 2 D gel image analysis software, Progenesis from nonlinear, these are just a very few number of software which I have mentioned, these are which are very commonly used, but there are many other good software also available which one can use to analyze these gels.

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Now, let me show you this animation for performing 2 dimensional gel analysis, how to step by step to analyze your gels.

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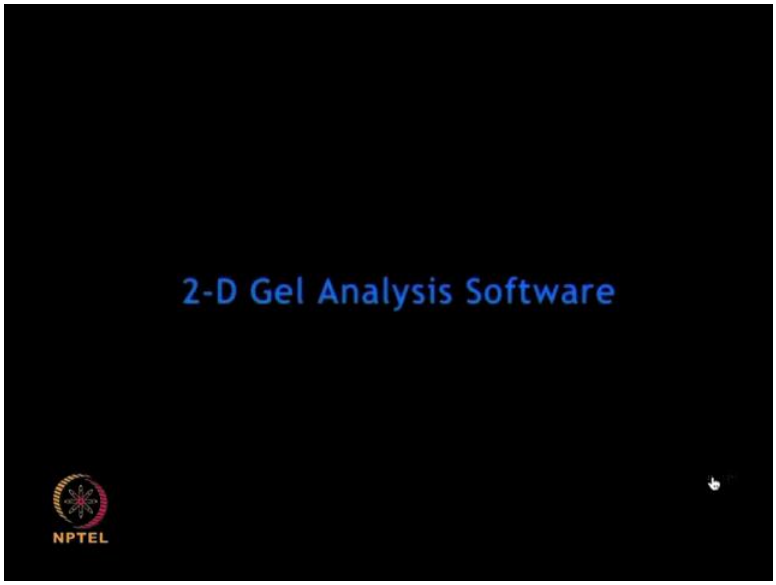

2 D gel analysis software, in this animation I will describe you how to analyze the 2 D gel images by using a generate software layout.

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So, first you need to load a gel image, it is possible to load either a single or multiple gel images simultaneously. This can be done by a means of the load option in the file menu, you can save the gel images and then you can crop the area depending upon, what area

you want to analyze. There are several tools which are available for the analysis of gel, it is possible to crop the gels by selecting a specific region that is to be studied and then selecting the crop gel function, cropping gel helps in selection of selection of region with high spot density or to reduce the regions which contain high background stains with no spot.

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Zooming into a selected region, if you want to expand a specific region, you can use zoom tool, specific selected region of the gel can be zoomed into for viewing the spot more closely and for comparison of spots between the two gels, this is particularly useful for gels with large number of spots.

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Once you have seen the area, you would like to overlay the images, overlaying of images is a particularly useful tool for the comparison of twogel, the gels are over laid such that they appear merged and spots that coincides will overlap with each other. This is extremely helpful while comparing the large clinical samples of controls and treatments so that you can obtain the clear indication of the proteins which are differentially expressed.

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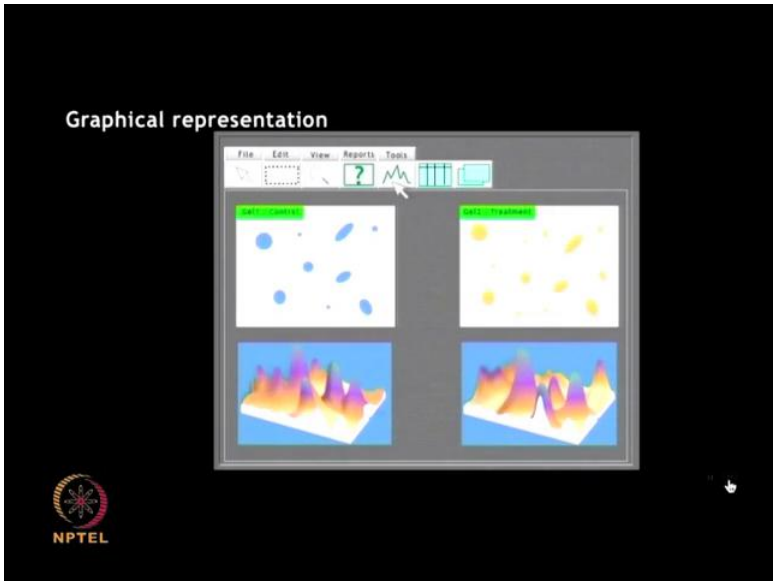

Now, after the analysis, one can look at the graphical representations of these three dimensional view of the spots, the spots on the gels can be displayed as three dimensional graph, either the entire gel can be chosen or a particular region can be selected for this representation, the peaks obtained in the graphical representation are directly related to the spot intensity.

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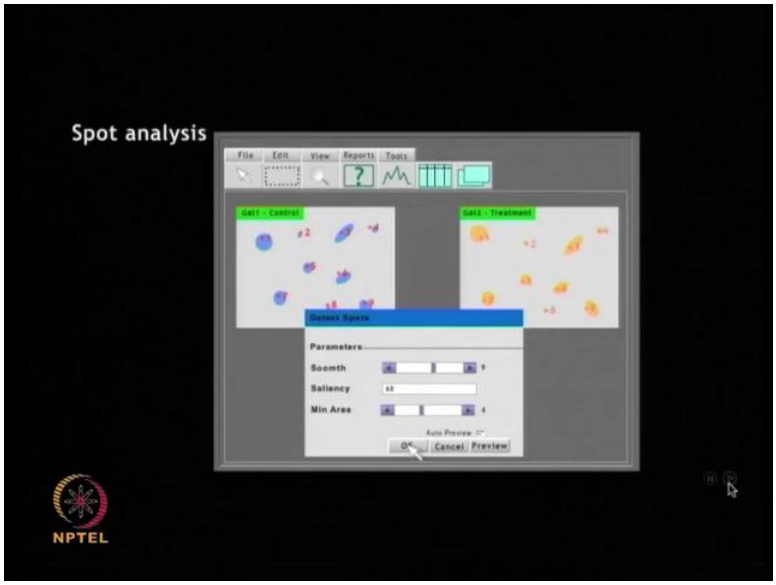

Next we will talk about spot analysis,every spot of the gel can be detected by selecting the detect spot option, various parameters such as smoothness, saliency and minimum area must be suitably adjusted for maximum clarity. Once this is done, each spot will either be encircled or marked with a cross, depends upon the setting along with the spot numbers.

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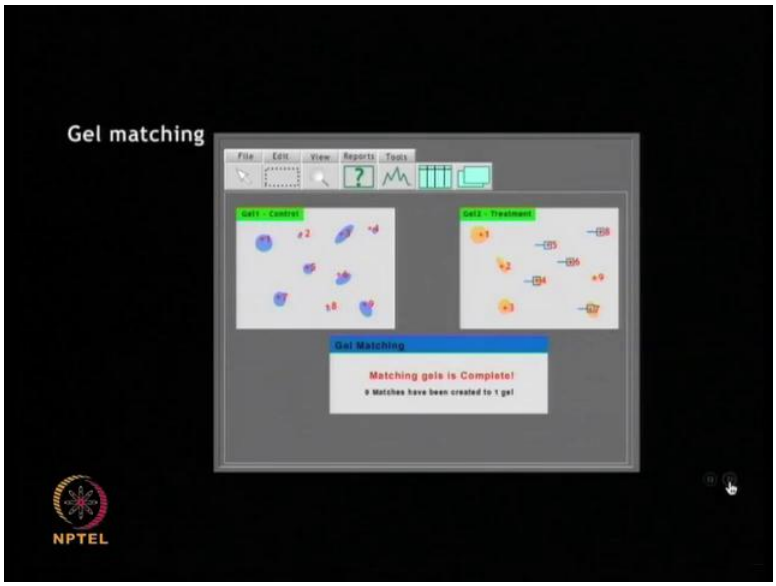

Now, I will describe you the gel matching, the software facilitates interpretation of the gel images by matching two different gel images which were obtained in your experiment. The matching spots are marked and after matching is done, any variation in the spot intensity, spot positions can be indicated by the blue lines as shown in the animation, and this provides an understanding about the reproducibility across the gels.

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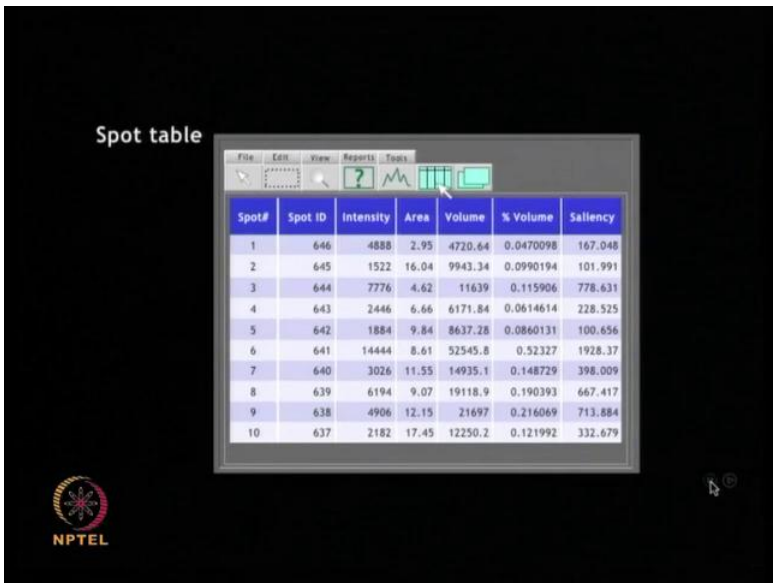

Once you have analyzed the gel, one can obtain the detailed information for these spots from spot table information regarding various physical parameters of each spot can be

obtained via this spot table which provides spot number, intensity, area and volume of the spots as well as the saliency of spots, these parameters help to judge the quality of a gel.

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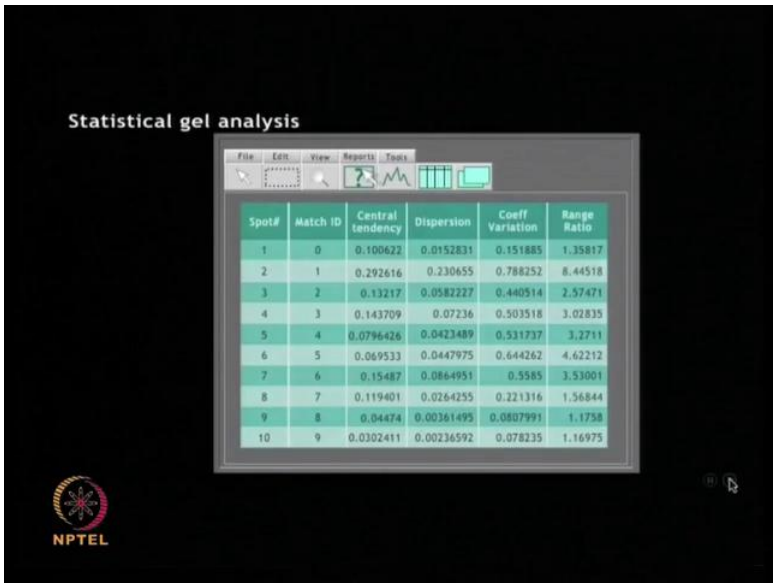

In addition to the physical parameters, various statistical parameters can also be computed for each gel and each spot on the gel such as central tendency, mean, median, dispersion, coefficient of variation standard deviation or other statistical parameters.

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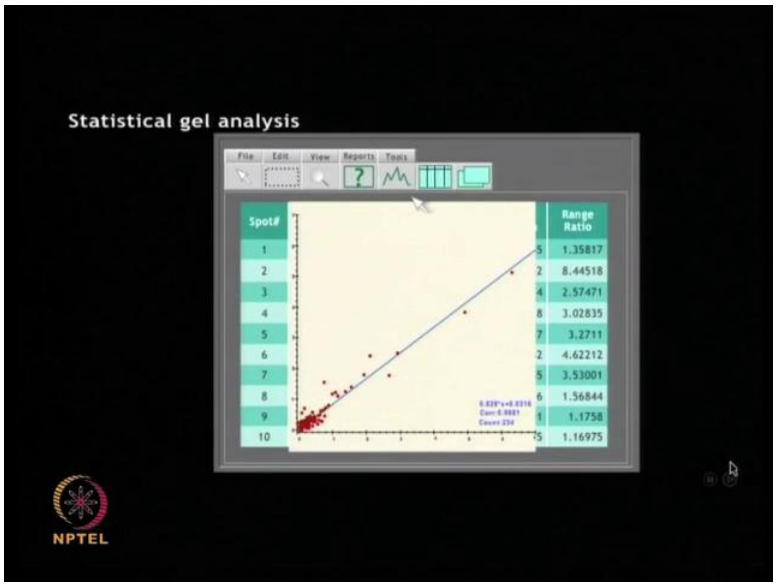

The scatter plots and histograms can also be plotted for clear data analysis, these can provide information regarding inter and intra gel variations.

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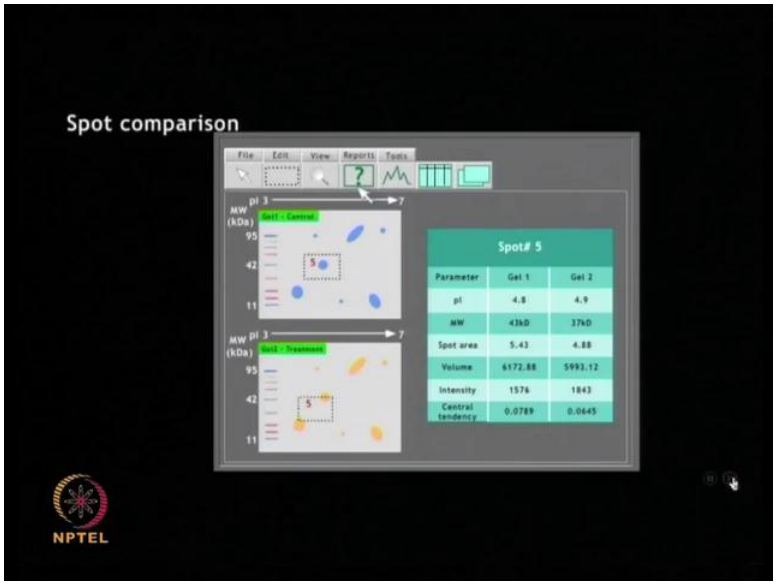

The spot comparison, it is possible to specifically compare a particular selected spot across the gels when the gel is run with molecular weight markers with molecular weight of unknown proteins can be estimated from this information. For example, as you seen in the animation; on the left hand side, you first loaded the molecular weight marker and now from that information, you can compute the information for the unknown protein to calculate its molecular weight and isoelectric point, these parameters, in addition to the other physical and statistical parameters can be obtained for each spot.

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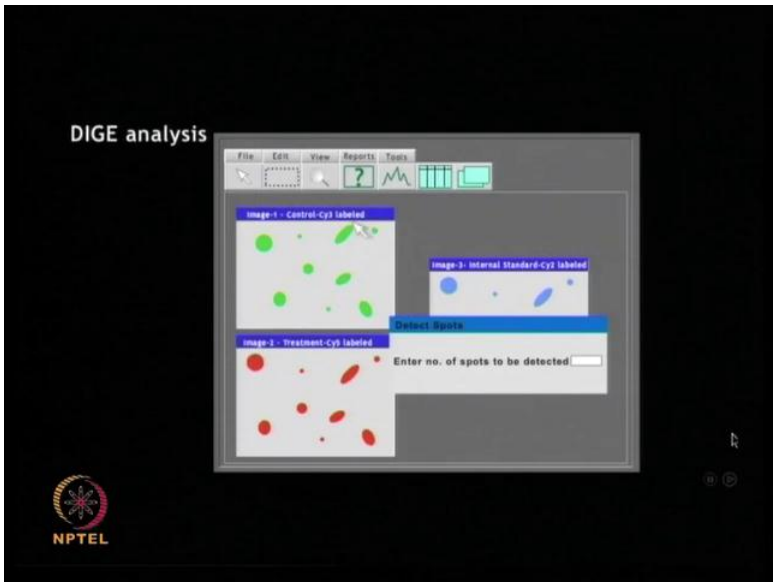

I will describe you a differentanalysis which is DIGE, D I G E, difference in gel electrophoresis, although we have very briefly touched upon the use of Cyanine dyes and I will describe you this technology in much more detail in the next lecture, but briefly I introduce here that DIGE analysis can be performed with a specialized software which can overlay the images obtained from the scanners for Cy3,Cy 5 and Cy 2 labeled samples. It can compare three gels simultaneously for which, one is typically the pooled internal standard containing all spots labeled with Cy 2 and you control and treatments are labeled with either Cy 3 or Cy 5.

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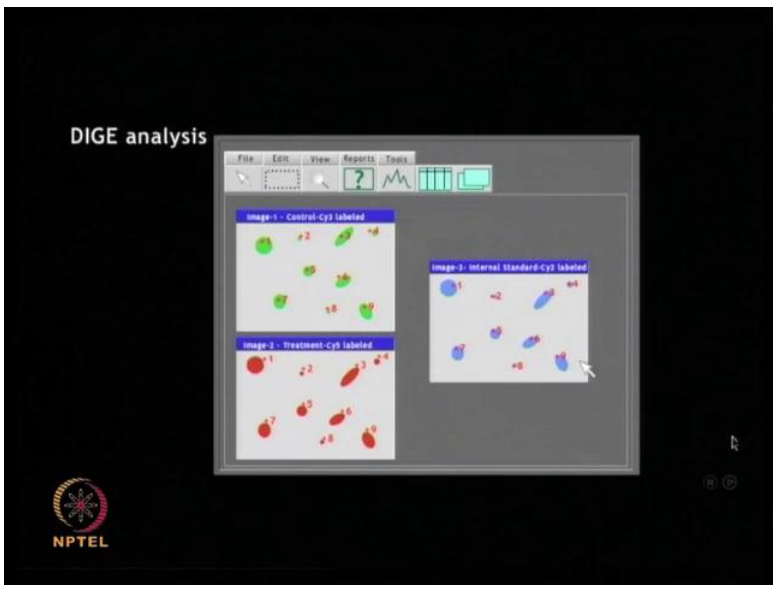

Any changes implemented in one gel such as cropping, its part selection, etcetera will be implemented across all the threegels in DIGE.

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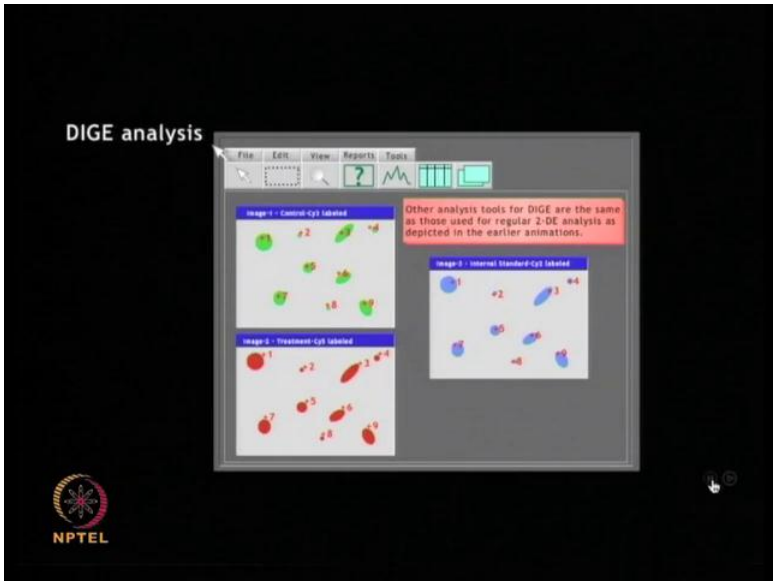

Other features and tools for DIGE analysis are similar to those used for 2 D E analysis, the physical and statistical parameters of all the spots on the gels can be determined through their corresponding reports.

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So, after looking atthis animation, now you are clear that you can do the comparison of your control and your treatment gels and you can identify few statistically significant

proteins which can be further used to do the spot picking and further mass spectrometry analysis. So, such a strip in this work flow is the spot picking, how to exercise your spot of interest from the gels.

Now, most of the time people use the comparison of the control and treatment gels which is abundance based proteomics, where you are looking at only change in the few proteins because of a given treatment or a given disease or a given condition. Now, if you want to compare this, then the number of protein changes which are going to happen overall is not going to be very much, may be you will have 50 to 100 proteins maximum which will alter their overall pattern, because of a given treatment. So, it is very easy if you have less number of protein spot, to excise this spot from the given gel even manually if you print this spot map or the 2 D gel image and keep it side by side along with your gel, it is easy and possible to locate those spot on the given gel and then you can excise those manually, you have to obviously ensure various quality control checks over there to avoid any contamination, but that can be done.

Now, other way of doing is to use a robot picker and exile the spot of interest that is more precise, but more costly. Many times when people want to know the global expression for all the proteins, for example for a given sample, for a given organism, if the proteome is unknown and you are the first one who want to characterize, identify all the proteins from that given sample, so probably you would like to know, what are all the proteins present in that given sample. In that case, you would like to identify all the proteins which are present on your gel, so you need to do the mass spectrometry for each of those protein spot that is not possible to do manually. So, in that case, you have to rely on using a spot picket.

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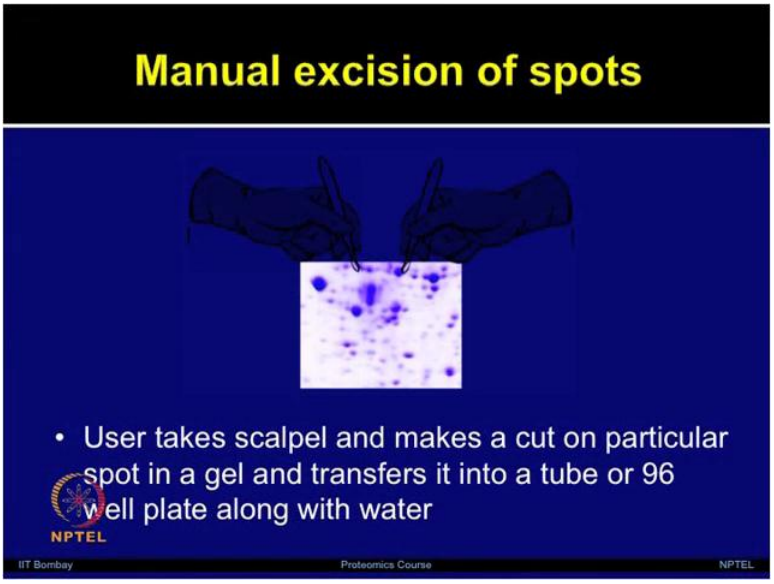

So, I will show you two methods here for the spot picking, one is the manual excision of the spot where user can take a scalpel and make a cut on a particular gel for the given spot of interest and then transfer that intoanother tube eppendorf tube, label that and then transfer it into the 96 well plate along with the water.

You have to ensure that you are using Millipore water or autoclaved, very purified form of the water, you are using a steriled samples, your glass plate on which you are keeping the gels are also very clean, you do not introduce any contamination, the hair or from your gloves or from any other dust particles available in environment, preferably do these steps in the sterile conditions in the laminar hood. By doing that you should be able to pick up the spots of your interest and transfer in the 96 well plate, further which can be used for doing other steps such as in gel digestion and mass spectrometry.

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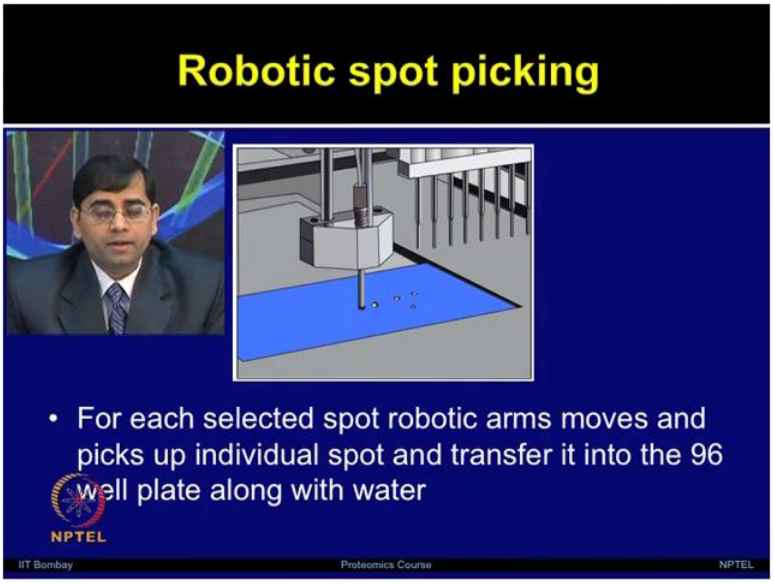

Now, second part is robotic spot picking, where as I mentioned that is more accurate, more precise, but it is more costly. If you are doing global profiling, you have to rely on the robot, robotic spot picking. Often if you are looking Cy dyes or some sort of fluorescence stains where you cannot even visualize the spots, so then also you have to rely on the robotic spot picking.

So, for each selected spot, the robotic arms moves and pick up the individual spot, now you can set up the x y quadrants in the software and then based on the location of the map or the spots present on the gel, it can go and exile the spots from the given gel, then it can be transferred to the 96 well plate for further processing. So, now, I have described you the work flow of performing two dimensional gel electrophoresis and in this work flow, today we have talked about staining methods as well as how to scan those images and analyze those images by using various software. After that you have completed the work flow of two dimensional electrophoresis, but I will try to teach you other gel based methods which can be employed to study the proteome.

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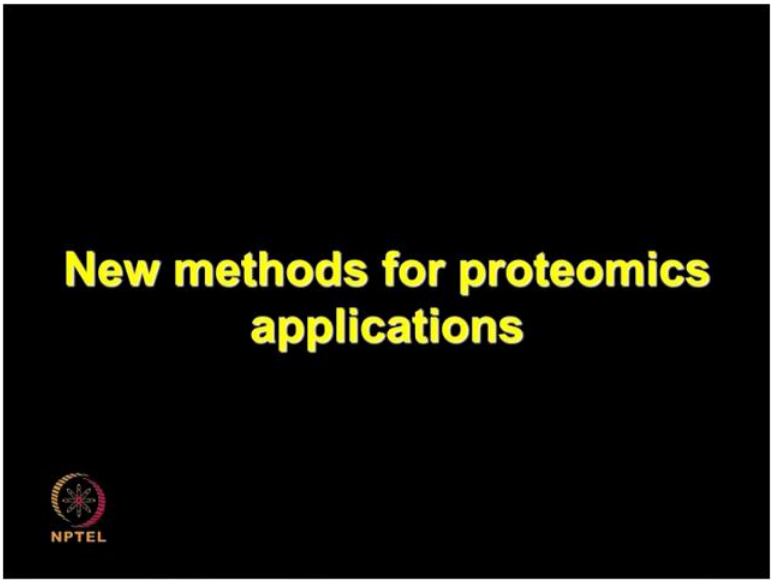

So, based on these gel based methods, there are new methods which have emerged for different proteomic applications.

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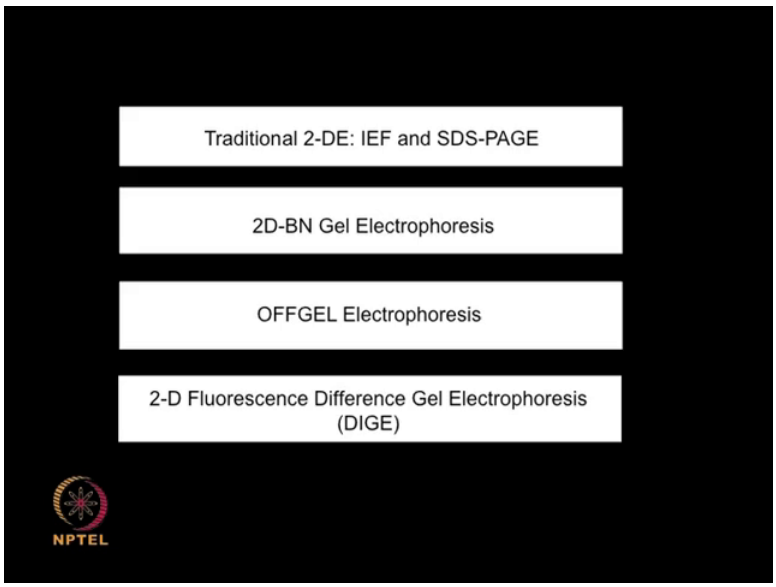

The conventional methods or traditional methods of twodifferent electrophoresis and SDS page, we have talked in detail, twodimensional blue native page electrophoresis, also we have described and discussed in the last lecture,off gel electrophoresis that is an another new technology introduced from Agilent that we will talk brieflyin this

lectureand 2 D fluorescence difference in gel electrophoresis or DIGE,this will be covered in the following lecture.

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So, let us briefly talk about off gel electrophoresis, as it the name suggests, in this method you are avoiding the separating proteins based on the gel. So, off gel electrophoresis can be performed on the immobilized pH gradient strip. But you do not need to use the gel approaches for separating the proteins and this method is mostly used for liquid chromatography based mass spectrometry applications, where people want to separate the proteins to reduce the complexity based on the isoelectric point, but you do not want to separate the proteins based on the gel for due to various artifacts,which gel based approaches can offer.

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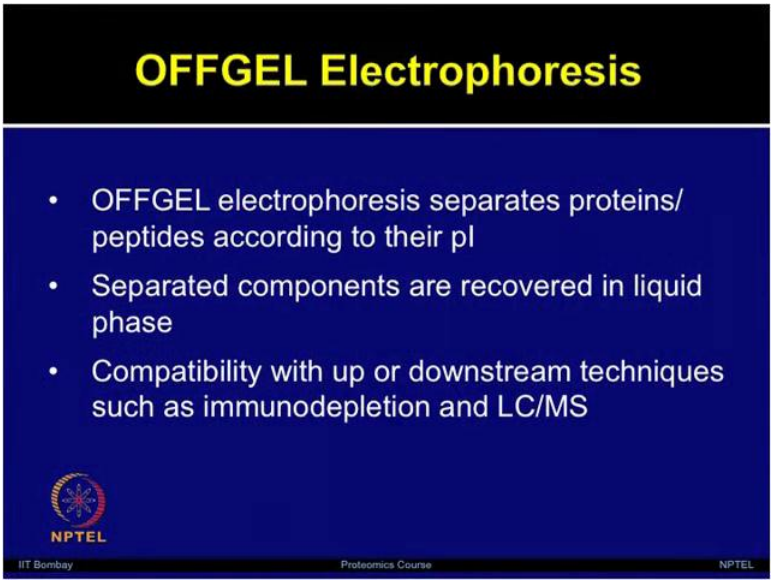

So, off gel electrophoresis, it separates proteins based on the isoelectric point of the proteins or peptides, we have talked about how to do isoelectric focusing in the previous lectures. Now, these separated components which are separated based on the pI values can be recovered in the liquid phase itself, while the i f has finished your protein samples are already present in the liquid phase and you can remove eliquotes in the different pH range so that you have the proteins separated based on the pI values in the liquid phase itself. Now, this method is very compatible for further downstream processing in LC and MS or doing immune precipitation.

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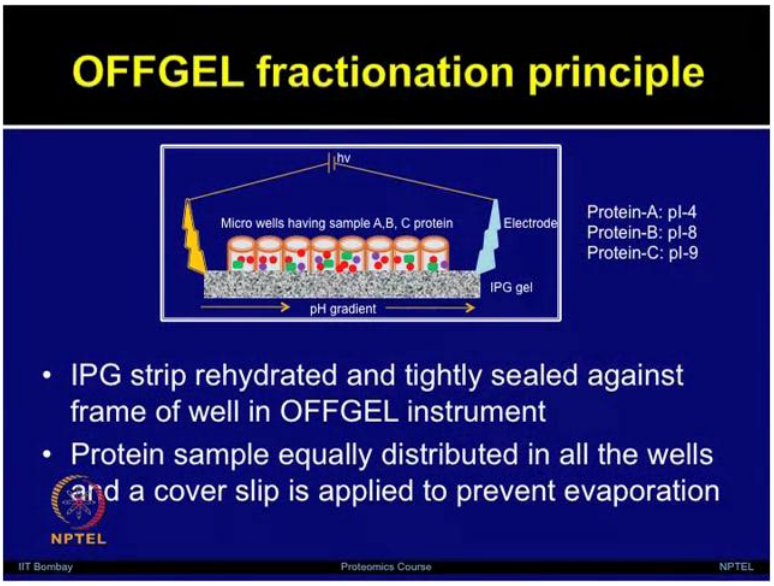

So, I will briefly mention you here, the fractionate on principle involved in off gel method where IPG strip can be rehydrated and tightly sealed against the frame of the well in off gel instrument. I am giving you example here for three proteins; let us say you want to separate protein A, protein B and protein C, each of these protein have different isoelectric points pI 4,8 and 9. Now, in the isoelectric focusing instrument, when you add your LPG strip, you apply your protein sample you have tighten the wells and now, protein samples will be equally distributed in the wells throughout on the IPG strip, there you need to apply a cover slip so that you can avoid any evaporation issues.

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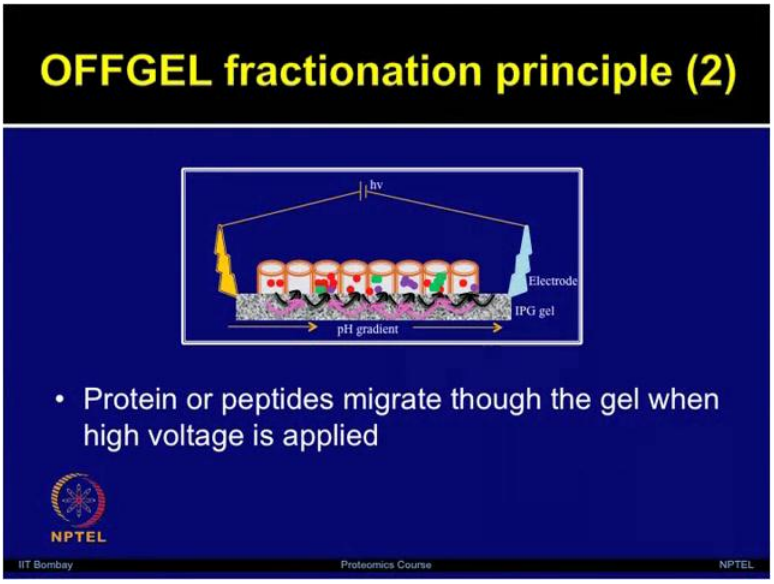

Now, the proteins and peptides in the electric field will migrate, because of the high voltage applied and proteins will start moving based on their isoelectric point.

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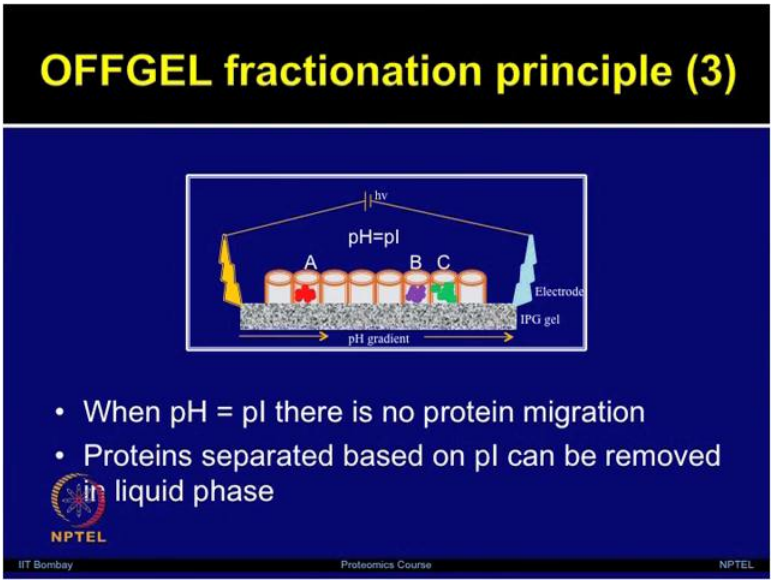

And when they will reach to a place where pH is equal to the isoelectric point, then there will be no further protein migration, now this is the protein separation which you wanted to happen. For example, A, B and C, you had threeproteins with different isoelectric point and now you wanted to separate those.

In this method, now you know in the pH range 4,8 and 9, you have those fractions which you can collect after doing the isoelectric focusing. So, proteins can be separated based on the isoelectric point and it can be removed in the liquid phase. Now, you have seen the very easy example of three proteins, ideally people use this for very complex protein mixtures to reduce the complexity of the overall proteome and separate or fractionate the overall proteome based on the isoelectric point, and collect each of the fractions which can be separately further analyzed using LC, MS approaches.

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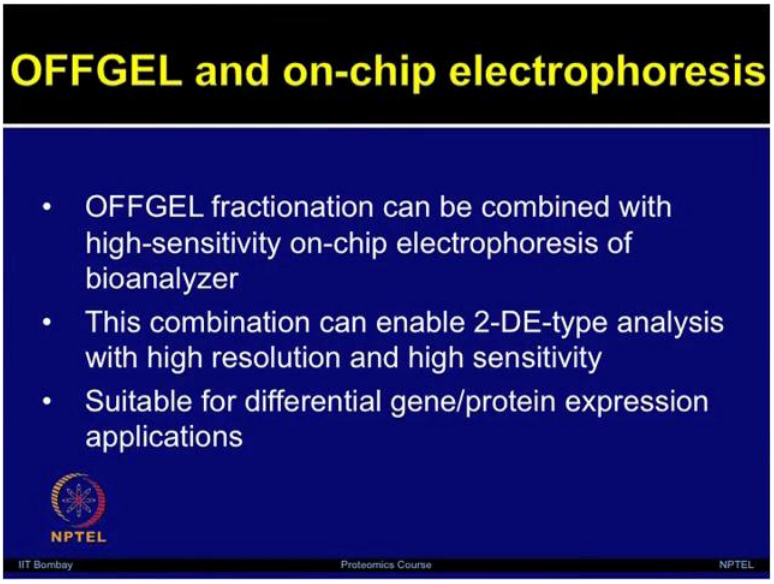

Now, offgel itself is sufficient and used widely for various LC, MS applications, but same manufacturer have also introduced another technology sothat one can separate the proteins in two dimensions, both in isoelectricpoint and the molecular weight. So, offgel and on chip electrophoresis method can be substituted for the gel based protein separation in the isoelectric point and the molecular weight.

Now, the offgel fractionation, it can be combined with the high sensitive on chip electrophoresis bioanalyzer which enables two dimensional electrophoresis type of analysis with very high resolution and high sensitivity, this method is very suitable for studying differential protein expression for different applications, so similar applications which one can do on two dimensional electrophoresis can also be performed by applying these twomethods together, the offgel and on chip electrophoresis.

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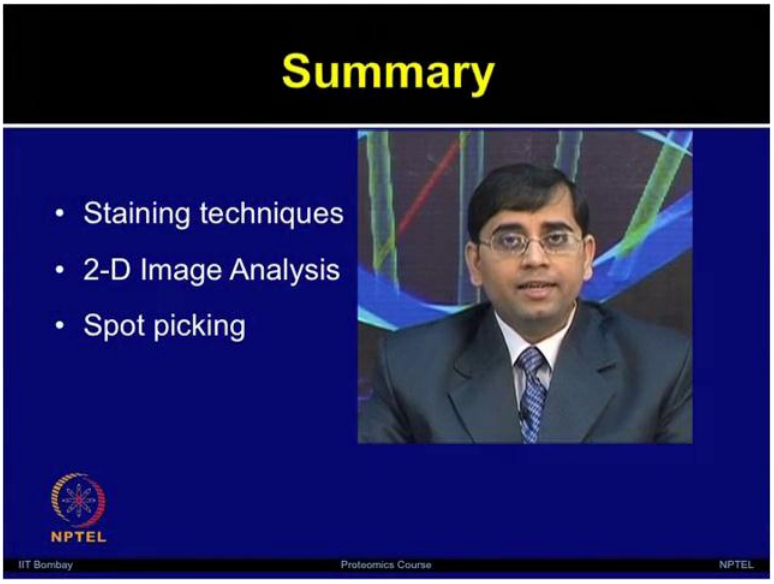

So, in summary,in today's lecture, we have talked about different type of staining techniques available which depending upon your application you can use, how to compare and analyze your two dimensional image obtained from different samples from controlled and treatments, how to excise the spots and then we had briefly looked at some variations or advanced forms of gel based proteomic methods such as offgel fractionation.We will continue this in the next lecture with more advanced form of electrophoresis such as twodimensional differences in gel electrophoresis.

I hope you are able to appreciate the various types of methods available to analyze the protein samples or the proteome, the complex proteome and how one can depending upon their challenging samples, apply different type of techniques. So, we will continue in the lecture, I hope you are able to appreciate the gel based proteomics and its potential. So, we will talk about 2 D DIGE in the next lecture, thank you.